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CpG Oligodeoxynucleotide and Montanide ISA 51 Adjuvant Combination Enhanced the Protective Efficacy of a Subunit Malaria Vaccine

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Unmethylated CpG dinucleotide motifs present in bacterial genomes or synthetic oligodeoxynucleotides (ODNs) serve as strong immunostimulatory agents in mice, monkeys and humans. We determined the adjuvant effect of murine CpG ODN 1826 on the immunogenicity and protective efficacy of the *Saccharomyces cerevisiae*-expressed 19-kDa C-terminal region of merozoite surface protein 1 (yMSP1$_{19}$) of the murine malaria parasite *Plasmodium yoelii*. We found that in C57BL/6 mice, following sporozoite challenge, the degree of protective immunity against malaria induced by yMSP1$_{19}$ in a formulation of Montanide ISA 51 (ISA) plus CpG ODN 1826 was similar or superior to that conferred by yMSP1$_{19}$ emulsified in complete Freund's adjuvant (CFA/incomplete Freund's adjuvant). In total, among mice immunized with yMSP1$_{19}$, 22 of 32 (68.7%) with ISA plus CpG 1826, 0 of 4 (0%) with CFA/incomplete Freund's adjuvant, 0 of 4 (0%) with CpG 1826 mixed with ISA (no yMSP1$_{19}$), and 0 of 11 (0%) with CpG 1826 alone were completely protected against development of erythrocytic stage infection after sporozoite challenge. The adjuvant effect of CpG ODN 1826 was manifested as both significantly improved complete protection from malaria (defined as the absence of detectable erythrocytic form parasites) ($P = 0.007$, chi square) and reduced parasite burden in infected mice. In vivo depletions of interleukin-12 and gamma interferon cytokines and CD4$^+$ and CD8$^+$ T cells in vaccinated mice had no significant effect on immunity. On the other hand, immunoglobulin G (IgG) isotype levels appeared to correlate with protection. Inclusion of CpG ODN 1826 in the yMSP1$_{19}$ plus ISA vaccine contributed towards the induction of higher levels of IgG2a and IgG2b (Th1 type) antibodies, suggesting that CpG ODN 1826 caused a shift towards a Th1 type of immune response that could be responsible for the higher degree of protective immunity. Our results indicate that this potent adjuvant formulation should be further evaluated for use in clinical trials of recombinant malarial vaccine candidates.

Malaria remains a leading cause of death among children under the age of 5 years in sub-Saharan Africa and one of the most devastating infectious diseases around the world (44). According to a World Health Organization report, malaria alone reduces the economic growth of Africa by more than 1% per year, adding up to hundreds of billions of dollars of lost income (34, 45). In spite of the availability of modern intervention tools, malaria incidence is still increasing around the world, primarily due to drug resistance by the parasite and mosquito resistance to commonly used insecticides. A vaccine that would reduce malaria-related mortality and morbidity offers hope in a deteriorating situation.

Of the more than 5,300 genes identified for the *Plasmodium falciparum* malaria parasite (17), only about 20 antigens are currently being developed for clinical trials. With the availability of the complete or partial genome sequences of several *Plasmodium* species (www.PlasmodB.org), we think that genomics-based antigen discovery will significantly increase the number of potential vaccine candidates. Several of the vaccine antigens identified by conventional methods induce significant degrees of protective immunity in experimental challenge models. However, in these experiments, the protective formulation required Freund's adjuvant or complicated primary and booster immunization regimens (16, 29). The effectiveness of primary and booster immunization regimens in humans is not proven, and R,TSS, the most effective recombinant protein-based vaccine tested, offered only 50% protection for a short duration (38). The discrepancy in the efficacies of vaccine antigens observed between experimental models and in clinical trials is primarily attributed to the lack of an effective adjuvant(s) that is safe for use in humans.

Merozoite surface protein 1 (MSP1), a 190- to 230-kDa protein present on the surface of all known *Plasmodium* spp., is one of the most studied malarial antigens. Precursor MSP1 undergoes several proteolytic processing events to produce at

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least four distinct fragments. The carboxyl-terminal 42-kDa ($MSP1_{42}$) fragment is further processed into two fragments of approximately 28 kDa and 19 kDa (2). Studies performed over the last several years have established that $MSP1_{42}$ and $MSP1_{19}$ are targets of protective immune responses against asexual stage parasites. Monoclonal antibodies generated against parasites produced MSP1 that recognizes epitopes on $MSP1_{19}$, and polyclonal antibodies generated by immunization with parasite-produced or recombinant $MSP1_{42}$ block the invasion of *P. falciparum* merozoites in in vitro cultures (1, 7) and reduce asexual stage *P. yoelii* parasite burden in passive transfer experiments in mice (12).

In the *P. falciparum-Aotus* challenge model, immunization with parasite-produced MSP1 (37), recombinant $MSP1_{42}$ expressed in baculovirus (6) or mammalian cells (39), or $MSP1_{19}$ expressed in *Saccharomyces cerevisiae* (26) when delivered in complete Freund's adjuvant (CFA) induced partial protection in *Aotus* monkeys against asexual stage parasites. Protective immunity required Freund's adjuvant; immunization in other adjuvant formulations failed to induce protection (28). In the murine *Plasmodium yoelii* model, *Escherichia coli*-produced $MSP1_{42}$ and $MSP1_{19}$ (11, 32, 40) and *S. cerevisiae*-produced $MSP1_{19}$ (21) protected mice against asexual stage parasite challenge.

Unmethylated CpG dinucleotide motifs present in bacterial DNA or as synthetic oligonucleotide sequences (ODNs) are strong stimulators of immune responses in mammalian hosts. These DNA sequences stimulate the immune system through a specific receptor, TLR9, which is expressed in humans and mice in B cells and plasmacytoid dendritic cells (25). The immune effects of CpG include direct triggering of B cells causing proliferation and nonspecific immunoglobulin synthesis, or synergistic enhancement of specific immunoglobulin synthesis in the presence of stimulation through B-cell antigen receptor (24), and activation of the monocytes, macrophages, and dendritic cells to produce high levels of a variety of cytokines (23). These elevated cytokines then stimulate NK cells to secrete gamma interferon (IFN-γ) and demonstrate increased lytic activity (10, 23). These immunostimulatory properties have made CpG ODNs a new class of adjuvants with effects ranging from non-antigen-specific therapy of allergens and cancers to immune enhancement of synthetic peptide- and recombinant protein-based vaccines.

We investigated the adjuvant effect of murine CpG ODN 1826 on the vaccine efficacy of *P. yoelii* $MSP1_{19}$ ($yMSP1_{19}$) expressed in *S. cerevisiae*. To accomplish this, we immunized C57BL/6 mice with $yMSP1_{19}$ plus CpG ODN 1826 emulsified in Montanide ISA 51 (ISA), and protective efficacy was determined based on its ability to protect against the development of erythrocytic stage malaria following challenge with *Plasmodium yoelii* sporozoites. The efficacy of this vaccine was compared to that of the "gold standard" formulation, $yMSP1_{19}$ delivered in CFA.

We also investigated the roles of antibodies and cellular responses in immunity induced by $MSP1_{19}$ delivered in CpG ODN plus ISA. To determine antibody-dependent immune mechanisms, we measured enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent-antibody test (IFA) immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, and Ig3 responses in pre- and post-sporozoite challenge immunized sera. For cellular immunity, we performed in vivo depletions of IFN-γ and interleukin-12 (IL-12) cytokines and $CD4^+$ and $CD8^+$ T cells by injections of antibodies specific to these cytokines and T cells.

MATERIALS AND METHODS

Mice. Four- to six-week-old C57BL/6 (*H-2*^{b}) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in the animal facility at the Naval Medical Research Center, Bethesda, Md. The mouse housing and experimentation conditions were in conformation with the principles set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

Recombinant $yMSP1_{19}$. The C-terminal 19-kDa fragment of the *P. yoelii* merozoite surface protein 1 ($yMSP1_{19}$) was produced as a secreted His_6-tagged recombinant protein in *S. cerevisiae*. The procedure for plasmid construction, expression, and purification of $yMSP1_{19}$ has been described earlier (40).

Adjuvants. CFA and incomplete Freund's adjuvant were purchased from Sigma Chemicals (St. Louis, Mo.). ISA, a water-in-oil emulsion, was purchased from Seppic Inc. (Fairfield, N.J.). ODNs were synthesized in the laboratory. Two CpG ODNs were used: 1826, TCCATGA*CG*TTCCTGA*CG*TT (CpG dinucletotides italic), and control 1982, TCCAGGACTTCTCTCAGGTT.

Immunizations. The following vaccine formulations were used in this study: $yMSP1_{19}$, 20 μg of $yMSP1_{19}$ in 50 μl of phosphate-buffered saline; CFA, 50 μl of CFA or incomplete Freund's adjuvant; ISA, 50 μl of Montanide ISA 51; ODN 1826, 28 μg of CpG ODN 1826; and ODN 1982, 28 μg of control CpG ODN 1982. Each mouse received three immunizations with 100 μl of vaccine per dose delivered by the subcutaneous route at 3-week intervals. Serum samples were taken from each mouse at the time of each immunization, 2 days prior to sporozoite challenge, and 28 days after the parasite challenge.

IgG isotype ELISA. ELISA was used to measure anti-$MSP1_{19}$ IgG or IgG isotype IgG1, IgG2a, IgG2b, and IgG3 titers in $MSP1_{19}$-immunized and control immunized mice. Antibody titers were measured in pre- and post-sporozoite challenge sera. Briefly, 96-well ELISA plates were coated with 100 μl of $yMSP1_{19}$ at 1 μg/ml by overnight incubation at 4°C. The plates were washed three times with 0.05% Tween–phosphate-buffered saline and then blocked for 1 h at 37°C with 1% bovine serum albumin–phosphate-buffered saline. The supernatants were discarded and 100 μl of 10-fold dilutions of samples of test sera were added to triplicate wells. Following three washes, 100 μl of an appropriate dilution of anti-mouse IgG conjugated to alkaline phosphatase (Promega Inc., Madison, Wis.) was added.

For isotype determination, 100 μl of an appropriate dilution of alkaline phosphatase-conjugated anti-mouse IgG1, IgG2a, IgG2b, or IG3 specific antibodies (PharMingen, San Diego, Calif.) were added. After incubation for 1 h at 37°C, plates were washed three times, phosphatase substrate tablets (Sigma Chemical Co.) were diluted in diethanolamine (Pierce Scientific, Rockford, Ill.), and 100 μl was added to each well; the reaction was allowed to proceed at room temperature. The optical density values for plates were read at 410 nm with an ELISA reader. The ELISA values shown are calculated values determined as interpolated titers at an optical density of 0.5.

IgG isotype IFA. An IFA was used to measure IgG, IgG1, IgG2a, IgG2b, and IgG3 responses against asexual stage *P. yoelii* parasites. Briefly, the IFA test was done on 12-well toxoplasmosis slides (Bellco Glass, Vineland, N.J.). Twofold dilutions of test sera were added to *P. yoelii* parasites on wells, and the wells were incubated for 1 h at 37°C. Following the three washes, wells were incubated with anti-mouse IgG or IgG isotype IgG1, IgG2a, IgG2b, or IgG3 antibodies conjugated to fluorescein isothiocyanate (Southern Biotechnology, Birmingham, Ala.). The slides were read with a fluorescent light microscope.

Antibodies. In vivo depletions of IFN-γ and IL-12 cytokines and $CD4^+$ and $CD8^+$T cells in $yMSP1_{19}$-immunized mice were done by injections with monoclonal antibodies specific to the cytokine or T-cell subset. IFN-γ depletion was accomplished with a rat IgG1 anti-IFN-γ monoclonal antibody, XMG-6 (9). For IL-12 depletion, rat IgG2a monoclonal antibody C17.8 was used (46). GK.15, a rat monoclonal IgG2a (14), was used for $CD4^+$ T-cell depletion. Anti-CD8 antibody was a mouse monoclonal IgG2a, clone 2.43 (35). Purified rat immunoglobulin (Rockland, Gilbertsville, Pa.) was used for control antibody treatment.

Cytokine and T-cell depletions. In vivo depletions were performed with the antibody treatment schedules described below. In relation to antibody injection, sporozoite challenge was given on day 0. All antibody injections were given by the intraperitoneal route. For IL-12 depletion, 1 mg of monoclonal antibody C17.8 was given at 12 h prior to and 3 h after sporozoite challenge. For IFN-γ depletion, 1.0 mg of monoclonal antibody XMG-6 was given on days −2, −1, 0, and

TABLE 1. Experiment 1: immunization groups, geometric mean of maximum parasitemias achieved, and arithmetic mean day of maximum parasitemia in C57BL/6 mice[a]

Group	Vaccine	No. of mice	GM of maximum % parasitemia (95% CIs)	Mean day of maximum parasitemia (95% CIs)
A	$yMSP1_{19}$ + CFA/IFA	4	1.9 (0.005, 651)	15 (9.6, 20.3)
B	$yMSP1_{19}$ + ODN 1826 + ISA[c]	4	1.2 (0.000002, 1246728)	17.5 (−14.3, 49)
C	$yMSP1_{19}$ + ODN 1982 + ISA	4	16.8 (4.3, 67.3)	17.8 (9.8, 19.7)
D	ODN 1826 + ISA	4	29.5 (1.9, 459)	17.5 (7.2, 27.8)
E	$yMSP1_{19}$ + ODN 1826	4	69.1 (59.7, 80.1)	18.5 (15.7, 21.2)
F	ODN 1826	3	47.9 (37.8, 60.5)	18.3 (16.9, 19.7)
G	ODN 1982	4	58.9 (49.9, 69.4)	19.3 (16, 22.5)
H	Naive	4	58.9 (32.5, 106.5)	19.5 (16.2, 22.8)

[a] C57BL/6 mice were immunized by three subcutaneous injections given at 3-week intervals. Two weeks after the last dose, mice were challenged intravenously with 100 *P. yoelii* 17 XNL sporozoites. Data presented here show the geometric mean (GM) of the maximum parasitemia for each group achieved during follow-up with their 95% confidence intervals (CIs) and the arithmetic mean day upon which that maximum occurred. In group B, only two of four mice developed an erythrocytic stage infection.

+2. For CD4 depletion, 1.0 mg of monoclonal antibody GK1.5 was given on days −7, −6, −5, −4, −3, −2, and −1, and then injections were continued three times a week during the course of the study. For CD8 depletion, 0.5 mg of monoclonal antibody 2.43 was given on days −3, −2, and −1. Depletion of $CD4^+$ and $CD8^+$ T cells was confirmed by staining the spleen cells with CD4 or CD8 surface marker antibody conjugated to fluorescein isothiocyanate, and the results were analyzed by flow cytometry analysis. For control antibody, 1.0 mg of normal purified rat immunoglobulin was given on days −7, −6, −5, −4, −3, −2, and −1 and then continued three times a week during the course of the study.

Parasite challenge and enumeration of parasites. On day 14 after the third immunization, mice were injected intravenously with 100 *P. yoelii* 17 XNL sporozoites. Beginning on day 3 post-sporozoite challenge, thin blood films were taken everyday for the next 22 days by making a nick at tail bases. Blood smears were stained with Giemsa stain, and parasites were counted with a light microscope.

Statistical analysis. Repeated-measure analysis and one-way analysis of variance were performed with SPSS for Windows (version 8; SPSS Inc., Chicago, Ill.), while chi-square was performed with Epilnfo (version 6.04; CDC, Atlanta, Ga.).

RESULTS

Adjuvant effect of CpG ODN 1826 on $yMSP1_{19}$ vaccine. We tested the immune enhancement effect of murine CpG ODN 1826 on the $yMSP1_{19}$ vaccine emulsified in Montanide ISA 51 and compared this formulation to the gold standard, CFA, the most effective adjuvant to deliver recombinant malaria antigens in experimental models. In two independent experiments, the efficacy of different vaccines was tested based on their ability to eliminate or reduce the burden of asexual erythrocytic form parasites in C57BL/6 mice that had received three subcutaneous immunizations followed by intravenous challenge with 100 *P. yoelii* 17XNL sporozoites.

In the first experiment, the $yMSP1_{19}$ plus ODN 1826 plus ISA formulation was compared to $yMSP1_{19}$ delivered in CFA. Intravenous injection of naive C57BL/6 mice with 100 *P. yoelii* sporozoites caused infections in four of four mice by day 8 postchallenge (data not shown). These mice self-cleared their infections after reaching a maximum geometric mean parasitemia of 58.9% (Table 1). Among the immunization groups, all four of four mice that received $yMSP1_{19}$ plus CFA developed patent infections (Fig. 1A; Table 1). However, the protective effect of vaccine was obvious since these mice cleared their infections at a low peak geometric mean parasitemia of 1.9% (0.01%, 34.6%, 18.1%, and 2.0%, respectively). In comparison, two of four mice that received $yMSP1_{19}$ plus ODN 1826 plus ISA had complete immunity (absence of detectable parasites during the 22-day reported observation period). The other two mice that developed parasitemia cleared their infections at a peak geometric mean parasitemia of 1.2% (0.4% and 3.6%, respectively) (Fig. 1B; Table 1). All four of four mice immunized with $yMSP1_{19}$ plus ODN 1982 (control CpG) plus ISA developed patent infections and experienced a significantly higher peak geometric mean parasitemia of 16.8% (7.0%, 23.6%, 48.1%, and 10.1%, respectively) (Fig. 1C; Table 1).

The protective immunity observed in these mice was antigen specific and adjuvant dependent. Injections with $yMSP1_{19}$ plus ODN 1826 (without ISA), ODN 1826 plus ISA (without $yMSP1_{19}$), and ODN 1826 or ODN 1982 administered with the immunization regimens described in Materials and Methods offered no protection against sporozoite challenge. The maximum peak parasitemias experienced by mice in these groups were almost similar to those of naïve mice (Fig. 1D; Table 1). We used one-way analysis of variance with Tukey's HSD post hoc test to analyze differences in maximum geometric mean parasitemias achieved in all groups during the 22-day follow-up period (Table 1). Groups A and B had the lowest geometric mean maxima (1.9% and 1.2%, respectively) and were significantly different from groups C, D, E, F, and H naïve. During the entire follow-up period, group B parasite densities remained below those of all other groups including group A (except on day 10). These results show that $yMSP1_{19}$ vaccine, when delivered in the ODN 1826 plus ISA adjuvant combination, induced protective immune responses against malaria that were equivalent to those provided by the CFA-based formulation.

In the second experiment, following *P. yoelii* sporozoite challenge, all eight of eight naïve C57BL/6 mice developed erythrocytic stage infection and reached the geometric mean peak parasitemia of 50.5% (Table 2). Among the immunizations, $yMSP1_{19}$ plus ODN 1826 plus ISA (group A) induced the highest degree of protective immunity; only three of eight mice developed patent infection (Fig. 2A; Table 2). Furthermore, the three infected mice cleared their infections after experiencing only low-grade peak parasitemias (0.07%, 0.04%, and 6.0%, respectively; geometric mean, 0.26%). In comparison, the $yMSP1_{19}$ plus control ODN 1982 plus ISA (group B) vaccine offered a significantly reduced protective effect, and all eight of eight mice developed relatively higher degrees of parasitemia (range, 1% to 46%; geometric mean, 6.47%). Protec-

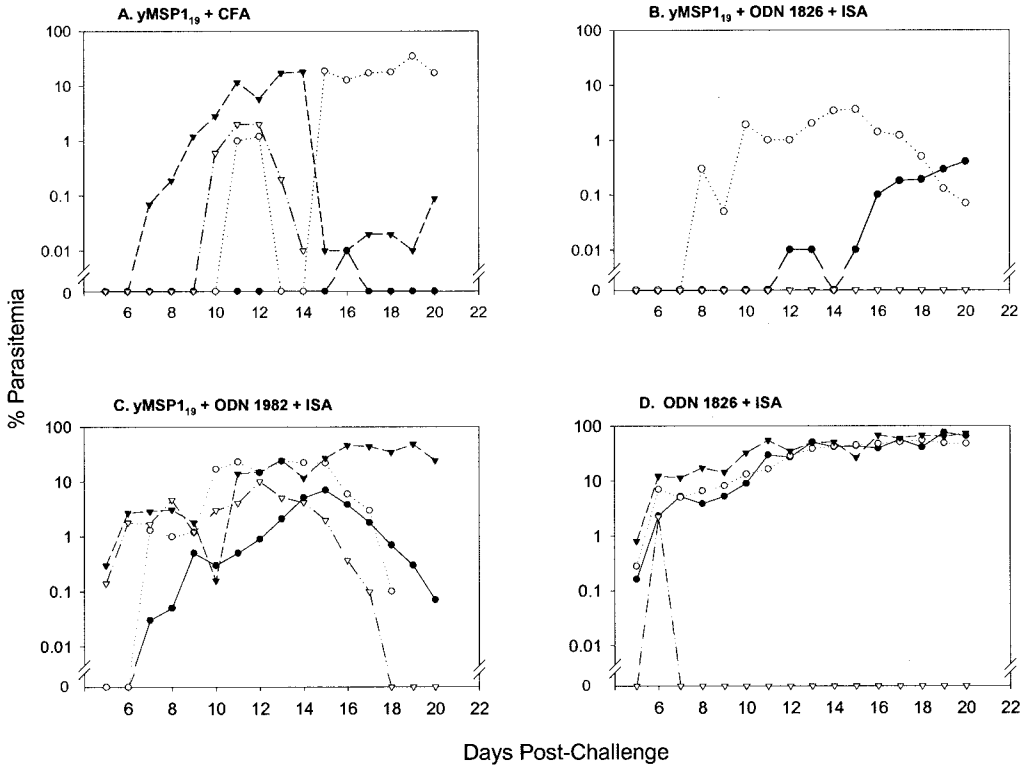

FIG. 1. Experiment 1. The course of parasitemias in C57BL/6 mice immunized by three subcutaneous injections with yMSP1$_{19}$ plus CFA (A), yMSP1$_{19}$ plus ODN 1826 plus ISA (B), yMSP1$_{19}$ plus ODN 1982 (control CpG) plus ISA (C), and ODN 1826 plus ISA (D). Two weeks after the last immunization, mice were challenged by the intravenous route with 100 *P. yoelii* 17 XNL sporozoites. Each group contained four mice.

tive adjuvant formulation required delivery in ISA 51 since MSP119 plus ODN 1826 had no antiparasitic effect (Table 2). Furthermore, three subcutaneous injections with ODN 1826 alone with the immunization schedule described above conferred no protection against sporozoite challenge (Table 2).

We used repeated-measure analysis with Tukey's HSD post-hoc test to analyze differences in parasitemias for groups A through D over the 22-day follow-up period (Table 2). The primary analysis showed that there were significant differences between the groups on some of the days ($P > 0.0009$). The post hoc analysis indicated that groups A and B consistently had significantly lower mean parasite densities than groups C and D.

We also compared groups based on complete protection. The groups were divided into two sets. Set 1 received yMSP1$_{19}$ plus ODN 1826 plus ISA (groups A, E, F, G, H, and I: Fig. 2 and Table 2) while set 2 received only portions of the combination vaccine (groups B, C, and D; Fig. 2B and Table 2). Set 1 had 20 mice completely protected and 8 not protected, while set 2 had none completely protected and 24 not protected ($P =$

TABLE 2. Experiment 2: immunization groups, geometric mean of maximum parasitemias achieved, and arithmetic mean day of maximum parasitemia in C57BL/6 mice[a]

Group	Vaccine	Antibody treatment[b]	n/N[c]	GM of maximum % parasitemia (95% CIs)	Mean day of maximum parasitemia (95% CIs)
A	yMSP1$_{19}$ + ODN 1826 + ISA	None	3/8	0.26 (0.0003, 234)	11.3 (9.9, 12.8)
B	yMSP1$_{19}$ + ODN 1982 + ISA	None	8/8	6.47 (0.86, 50.0)	15.7 (13.4, 18.1)
C	yMSP1$_{19}$ + ODN 1826	None	6/6	53.2 (45.1, 62.8)	17.5 (14.1, 20.9)
D	ODN 1826	None	8/8	66.6 (60.0, 73.9)	17.3 (15.7, 18.8)
E	yMSP1$_{19}$ + ODN 1826 + ISA	Anti-IL-12	3/4	0.027 (0.007, 0.1)	12.3 (7.2, 17.5)
F	yMSP1$_{19}$ + ODN 1826 + ISA	Anti-IFN-γ	1/4	63.3	16
G	yMSP1$_{19}$ + ODN 1826 + ISA	Anti-CD4	1/4	0.6	8
H	yMSP1$_{19}$ + ODN 1826 + ISA	Anti-CD8	0/4		
I	yMSP1$_{19}$ + ODN 1826 + ISA	Control Ig	0/4		
J	Naive	None	8/8	50.5 (39.6, 64.3)	16.7 (15.1, 18.3)

[a] See Table 1, footnote *a*.
[b] In groups E to I, after three immunizations, mice were injected intraperitoneally with antibodies specific to IL-12, IFN-γ, CD4$^+$ T cells, or CD8$^+$ T cells or a control antibody. For details of T-cell and cytokine depletions, see the Materials and Methods section.
[c] n/N, number of infected mice/total number of mice per group.

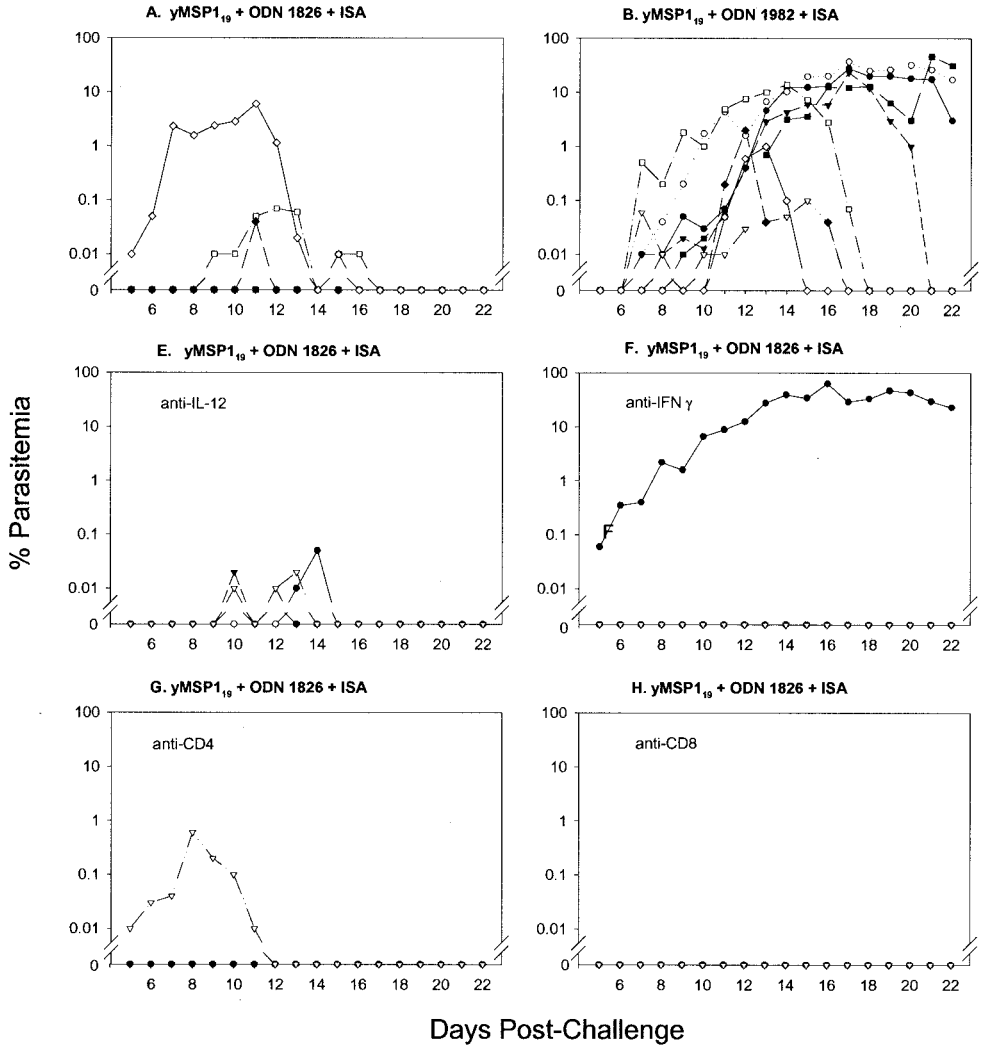

FIG. 2. Experiment 2. The course of parasitemias in C57BL/6 mice immunized by three subcutaneous injections with yMSP1$_{19}$ plus ODN 1826 plus ISA (A, E, F, G, and H), and yMSP1$_{19}$ plus ODN 1982 (control CpG) plus ISA (B). Two weeks after the last immunization, mice were challenged by the intravenous route with 100 *P. yoelii* 17 XNL sporozoites. In groups E to H, mice received treatment with antibodies specific to anti-IL-12 (E), anti-IFN-γ (F), anti-CD4 (G), and anti-CD8 (H). The detailed protocol for antibody treatment is described in Materials and Methods. Groups A and B contained eight mice per group, whereas groups E, F, G, and H each had four mice.

0.00003, chi square). We also compared protection in groups A and B. These groups differ only by CpG ODN (group A, ODN 1826; group B, control ODN 1982). Five of eight mice were completely protected in group A, while zero of eight mice were completely protected in group B ($P = 0.007$, chi square). These results clearly demonstrated that addition of ODN 1826 improved the protective efficacy of the yMSP1$_{19}$ plus ISA vaccine.

Cytokines and T cells in immunity. Having established that immunization with the yMSP1$_{19}$ plus ODN 1826 plus ISA vaccine either completely eliminated or caused a significant reduction in parasite burden, we next wanted to understand the immunological mechanisms that mediated protection. Since sporozoites were used as challenge inoculum, we examined the role of cytokines and T cells that mediate immunity against both the liver stage and erythrocytic forms of parasites. This was accomplished by in vivo depletion of IFN-γ and IL-12 cytokines and $CD4^+$ and $CD8^+$ T cells in mice that had received three immunizations with the yMSP1$_{19}$ plus ODN 1826 plus ISA vaccine.

Depletion of IL-12 was accomplished by two intraperitoneal injections with monoclonal antibody C17.8 (group E). For the depletion of IFN-γ, mice were given four intraperitoneal injections with monoclonal antibody XMG-6 (group F). Injections of anti-IL-12 had no significant effect on immunity. Following sporozoite challenge, one mouse had no patent infection while the other three of the four mice developed low-grade parasitemias (geometric mean, 0.027%; Fig. 2E and Table 2). The overall level of immunity observed in this group was not significantly different from that of the mice in the group A that received no anti-IL-12 antibody treatment. Surprisingly, following IFN-γ depletion, one mouse experienced a high-grade parasitemia while the other three mice remained solidly immune (Fig. 2F and Table 2). Since IFN-γ depletion had no effect on immunity in three of the four mice in the

group, absence of immunity in one mouse could be attributed to failed immunization. These results suggest that this immunity is not solely dependent upon IL-12 or IFN-γ. However, it is possible that these cytokines might still play a role in immunity either by acting in concert with other immune mechanisms or by influencing the type of immune response generated. The regimen of cytokine depletion used in this study has been shown to be effective in the elimination of IFN-γ- and IL-12-dependent immunity induced by immunization with irradiated sporozoites (15).

To determine the role of $CD4^+$ and/or $CD8^+$ T cells in immunity, mice were given intraperitoneal injections with GK1.5, an anti-CD4 monoclonal antibody (group G), or with 2.43, an anti-CD8 monoclonal antibody (group H), with the protocol described in Materials and Methods. Injections with rat anti-mouse immunoglobulin antibody served as a control (group I). Depletion of $CD4^+$ and $CD8^+$ T-cell populations was confirmed by flow cytometry analysis of splenic T cells taken from mice on the day of sporozoite challenge. Injections of GK1.5 and 2.43 antibodies eliminated >95% of $CD4^+$ and $CD8^+$ T cells (data not shown). Following sporozoite challenge, three of four mice in the CD4-depleted and four of four mice in the CD8-depleted groups had no detectable erythrocytic parasites in the circulation during the observation period (Fig. 2G and H). One infected mouse in the CD4-depleted group reached the peak parasitemia of 0.6%. Injections with control rat immunoglobulin had no effect on immunity; all four of four mice remained free of erythrocytic form parasites following sporozoite challenge (Table 2). These results suggest that immunity induced by $yMSP1_{19}$ plus CpG 1826 plus ISA in C57BL/6 mice was not mediated by the direct effector function of $CD4^+$ or $CD8^+$ T cells.

IgG and its isotypes in immunity. We next examined an association between anti-$MSP1_{19}$-specific IgG, IgGI, IgG2a, IgG2b, and IgG3 levels and protection against malaria, in particular the effect of the addition of CpG ODN on the $yMSP1_{19}$ plus ISA vaccine. Titers of total IgG and its isotypes were measured by ELISA against recombinant $yMSP1_{19}$ and by IFA against the erythrocytic form *P. yoelii* parasites.

In experiment 1, immunizations with $yMSP1_{19}$ plus CFA (group A) or with $yMSP1_{19}$ plus ISA delivered in combination with ODN 1826 or control ODN 1982 (groups B and C) induced high levels of ELISA IgG titers (geometric means, 80,702, 201,050, and 122,143, respectively; Fig. 3) in presporozoite challenge sera. Control vaccines (groups D, E, F, and G) induced significantly lower or nondetectable levels of IgG antibody responses (Fig. 3). Geometric mean ELISA titers (calculated from $\log_{10}$-transformed values) for immunization groups A through C were compared by one-way analysis of variance (Tukey's HSD post hoc test). This analysis revealed that IgG titers among the three groups were not significantly different.

In experiment 2, sera from groups A and B had high but nondistinguishable ELISA IgG titers (geometric mean, 606,144 and 784,755, respectively) (Fig. 4). Antibody levels for some of the ELISA IgG isotypes were different between groups A and B. Group A had a 7.2-fold-higher IgG2a response than group B (geometric mean, 107,103 and 14,832, respectively). No significant differences were seen in IgG1 (geometric mean, 598,825 and 637,529) and IgG2b (geometric mean, 60,021 and 48,148) levels between the two vaccine groups (Fig. 4).

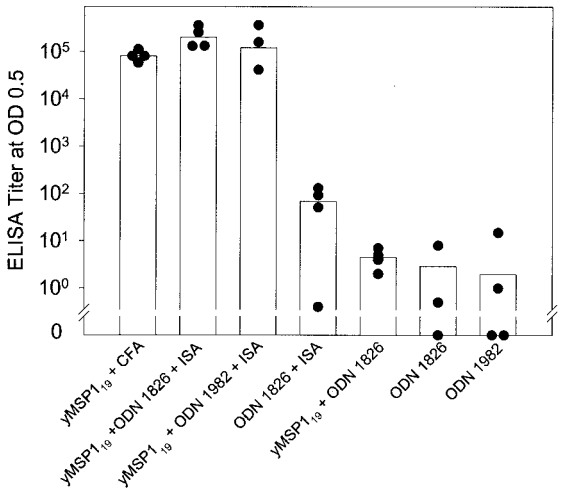

FIG. 3. Experiment 1. ELISA IgG responses in C57BL/6 mice immunized with $yMSP1_{19}$ plus CFA (A), $yMSP1_{19}$ plus ODN 1826 plus ISA (B), $yMSP1_{19}$ plus ODN 1982 (control CpG) plus ISA (C), ODN 1826 plus ISA (D), $yMSP1_{19}$ plus ODN 1826 (E), ODN 1826 (F), and ODN 1982 (G). ELISA titers shown are calculated values determined as interpolated titers at an OD of 0.5. Open bars represent geometric mean ELISA titers, and solid circles represent responses in individual mice.

In experiment 2, IFA IgG and isotype titers were measured against *P. yoelii* erythrocytic form parasites. Groups A and B had very high but comparable levels of IFA IgG titers (geometric mean, 729, 824, and 452,235, respectively) (Fig. 5). Similar to ELISA IgG isotypes, differences in IFA IgG isotype levels were noted between the groups. Compared to group A, sera from group B had 2.7-fold-higher IgG1 (geometric means,

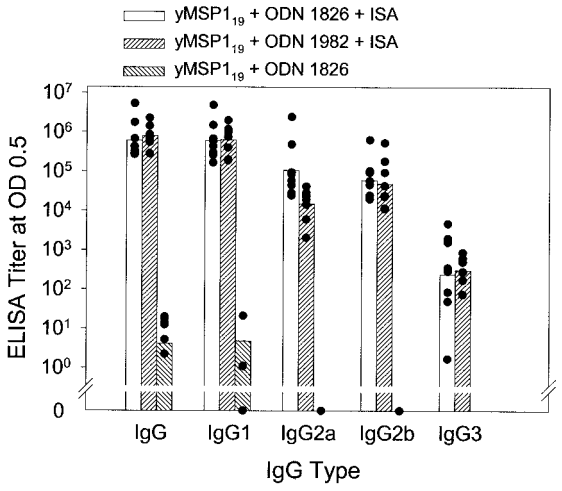

FIG. 4. Experiment 2. ELISA IgG, IgG1, IgG2a, IgG2b, and IgG3 responses in C57BL/6 mice immunized with $yMSP1_{19}$ plus ODN 1826 plus ISA (A), $yMSP1_{19}$ plus ODN 1982 (control CpG) plus ISA (B), and $yMSP1_{19}$ plus ODN 1826 (C). ELISA titers shown are calculated geometric mean values determined as interpolated titers at an OD of 0.5. Bars represent geometric mean ELISA titers, and solid circles represent responses in individual mice.

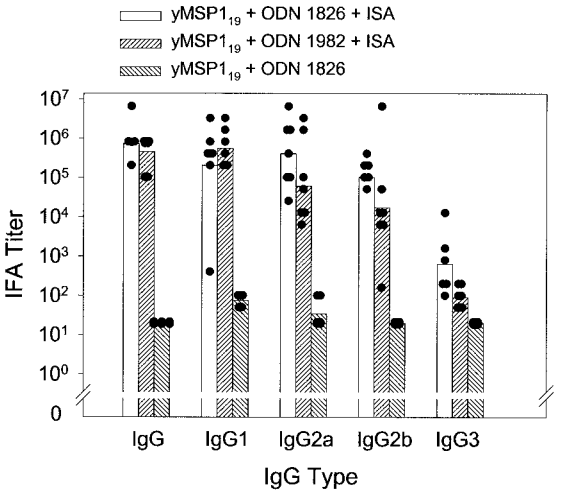

FIG. 5. Experiment 2. IFA IgG, IgG1, IgG2a, IgG2b, and IgG3 responses against asexual stage *P. yoelii* parasites determined in C57BL/6 mice immunized with yMSP1$_{19}$ plus ODN 1826 plus ISA (A), yMSP1$_{19}$ plus ODN 1982 (control CpG) plus ISA (B), and yMSP1$_{19}$ plus ODN 1826 (C). Bars represent geometric mean IFA titers, and solid circles represent responses in individual mice.

204,800 and 551,128) levels (Fig. 5). On the other hand, group A had 6.7-fold-higher IgG2a (geometric means, 409,600 and 60,887) and 5.8-fold-higher IgG2b (102,400 and 17,441) levels over group B. Both ELISA and IFA IgG3 levels were consistently low in all vaccinated groups (Fig. 4 and 5). A combined analysis of the ELISA and IFA IgG isotype titers revealed that addition of CpG ODN 1826 to the yMSP1$_{19}$ plus ISA vaccine led to the induction of higher IgG2a and IgG2b (Th1 type) responses whereas mice that received control CpG ODN 1982 had higher IgG1 (Th2 type) responses. The ELISA and IFA IgG and its isotype levels in postsporozoite challenge sera (taken 28 days after parasite challenge) were generally similar to the levels in the prechallenge sera, suggesting that parasite infection had no significant boosting effect in yMSP1$_{19}$ immunized mice (data not shown).

DISCUSSION

Recent availability of the complete and partial genome sequence information for several *Plasmodium* spp. has opened the possibility of rapid discovery of new vaccine antigens. Experience from the almost two decades of malaria vaccine research has shown that the preclinical phase of malaria development (antigen identification, characterization, and the availability of clinical-grade vaccine) is tedious, costly, and time-consuming. Several recombinant vaccine antigens when first tested in experimental models showed promising results. However, further testing of the same antigens in humans produced less encouraging results. None of the malaria vaccine formulations tested so far has induced a sufficient degree of protective immunity to warrant its production as a licensed vaccine. One major factor attributed to the superior vaccine efficacy observed in animal models is the presence of CFA in protective formulations. Many malaria vaccine researchers believe that the identification of a new potent adjuvant(s) safe for human use would greatly accelerate malaria vaccine development. Although there is a rationale to invest in research towards genomics-based protective antigen discovery, a need still remains to optimize the immunogenicity of the malaria vaccine antigens currently under development.

Previously, we and other groups have demonstrated that immunization with recombinant MSP1$_{42}$ or MSP1$_{19}$ induced protection in mice (11, 32, 40, 41) and monkeys (8, 26, 39) against challenge with erythrocytic form malaria parasites. In monkeys, protective formulation required CFA; immunization in other adjuvants induced only partial or no protection (28). In this report, we show that *S. cerevisiae*-expressed *P. yoelii* MSP1$_{19}$, when delivered in Montanide ISA 51 and murine CpG ODN 1826, induced either complete protection from malaria or a significantly reduced parasite burden in C57BL/6 mice following sporozoite challenge (Fig. 1B and 2A; Tables 1 and 2). Addition of CpG ODN 1826 had a measurable immunostimulatory effect on this immunity. Data combined from two experiments showed that 22 of 32 (68.8%) mice immunized with yMSP1$_{19}$ plus ODN 1826 plus ISA were completely protected from malaria. In comparison, none of the mice that received yMSP1$_{19}$ plus control ODN 1982 plus ISA had complete protection and 12 of 12 mice in this group experienced high-grade parasitemias before self-resolving their infections (Fig. 1C and 2B; Tables 1 and 2). Optimal vaccine formulation required the presence of yMSP1$_{19}$ plus Montanide ISA 51 and ODN 1826; yMSP1$_{19}$ plus ODN 1826 without ISA, ODN 1826 plus ISA, or ODN 1826 alone failed to induce any protection. These results show that the immunostimulatory effect of ODN 1826 was antigen specific and that induction of protective immune responses required emulsion in Montanide ISA. It is also important to note that the degree of protective immunity induced by the yMSP1$_{19}$ plus ODN 1826 plus ISA vaccine was comparable to that conferred by the gold standard vaccine formulation, yMSP1$_{19}$ delivered in CFA (Fig. 1A versus B).

Unmethylated CpG dinucleotide sequences present in bacterial genomes or as synthetic CpG ODNs possess the unique property to cause strong immune stimulation in mammals by a variety of mechanisms. These DNA sequences directly act upon B cells to cause proliferation and polyclonal immunoglobulin synthesis and at low CpG ODN concentrations promote antigen-specific immune responses by synergistically acting in concert with B-cell antigen receptors (24). CpG ODNs also cause stimulation of monocytes, macrophages, and dendritic cells to express increased levels of costimulatory molecules and to secrete high levels of certain cytokines such as IFN-γ and IL-12 (23). These cytokines then activate NK cells for enhanced IFN-γ synthesis and lytic activity (10). CpG ODNs also stimulate monocytes to mature into functional dendritic cells that support antigen-specific humoral and cellular responses (20). These observations were consistent with reports that injection of CpG DNA alone rendered protection against a variety of allergens and infectious agents by non-antigen-dependent mechanisms (3, 19, 43) and also enhanced the protective effects of antigen-specific immunity (13, 22, 33, 42).

We have earlier demonstrated that treatment of mice intramuscularly with ODN 1826 without any parasite antigen conferred short-term protection from malaria following challenge with *P. yoelii* sporozoites (19). The highest degree of protection

was observed when CpG ODN was administered 1 or 2 days prior to challenge. The protection was dependent on IFN-γ and IL-12, suggesting that CpG ODN administration activated an innate immune response leading to nonspecific secretion of Th1 type cytokines (19). The CpG ODN-mediated protection achieved in the absence of parasite antigen depended upon the time and route of ODN delivered. In the present study, no protection from malaria was observed in mice that had received three subcutaneous administrations of ODN 1826 given at 3-week intervals followed by sporozoite challenge 2 weeks later (Tables 1 and 2). In another prior study, addition of CpG ODN in yMSP1$_{19}$ formulated with alum enhanced antigen-specific IgG production and induced partial protection in mice against lethal *P. yoelii* erythrocytic form parasites (33). Although this vaccine formulation prevented death against a virulent parasite challenge, all mice experienced high levels of parasitemias before clearing their infections (33).

Understanding the mechanisms of protective immunity induced by natural infection, attenuated parasites, or recombinant produced vaccines is important to design the next generation of malaria vaccines. Research performed over the last several years has demonstrated that in the murine *P. yoelii* model, irradiated sporozoite vaccine-induced immunity against the preerythrocytic stage parasites was mediated by CD8$^+$ T cells, IFN-γ, and IL-12 (15, 36). On the other hand, antibodies, CD4$^+$ T cells, and IFN-γ participated in the clearance of primary infection and the acquisition of reinfection immunity against the erythrocytic form parasites (27, 31). Immunity induced by yMSP1$_{19}$ plus CFA vaccine has been shown to be primarily an antibody-mediated one that targeted erythrocytic form parasites (12, 21, 40). In these experiments, erythrocytic form parasites were used as the challenge inoculum. We chose sporozoite challenge since it is better reflective of human exposure in the field. However, this choice added the possibility that the vaccine-induced anti-MSP1 immunity observed here could also have been directed against late liver stage parasites that expressed MSP1 (30).

Our results show that in vivo depletions of IL-12 and IFN-γ cytokines and CD4$^+$ and CD8$^+$ T cells in mice immunized with yMSP1$_{19}$ plus ODN 1826 plus ISA had no significant influence on immunity (Fig. 2E, F, G, and H). This finding discounts the possibility that any of these particular cytokines and T cells functioning through cellular mechanisms were the primary contributors of immunity. However, it still remains possible that either one or a combination of these immune factors at least partially contributed towards immunity.

We find a direct correlation between the levels of IgG isotypes and protective immunity. Sera from the group of mice that showed the best protection also had the highest ELISA IgG2a and IgG2b titers. In contrast, mice that received control ODN 1982 had higher levels of IFA IgG1 antibodies. These results suggest that inclusion of CpG ODN 1826 in the vaccine formulation caused a shift towards a Th1 type immune response that appears to be responsible for the enhanced protective immunity observed. In earlier experiments, higher IgG1 and IgG3 (Th2 type response) levels correlated with protection induced by yMSP1$_{19}$ plus CFA vaccine (21, 40).

The safety, tolerability, and adjuvant effect of Montanide and CpG ODN have been determined in preclinical studies and in humans. Montanide ISA 720 has been used in clinical trials to enhance the immunogenicity of synthetic peptides and recombinant proteins against tumor antigens (5) and several infectious diseases, including malaria (18). Similarly, the adjuvant effect of CpG ODNs was determined in preclinical studies for the treatment of allergens (3), to enhance the immunogenicity of recombinant antigens (13, 22, 33) or as monotherapy for cancers (4, 25, 42).

In conclusion, our results show that the protective adjuvant effect of CpG ODN 1826 plus Montanide ISA 51 formulation on yMSP1$_{19}$ vaccine was comparable to that offered by CFA. The precise nature of the protective immune response induced by this vaccine remained unclear, but a Th1 type antibody response appeared to play a dominant role. However, a potential role for cellular immune mechanisms is also possible. Our results warrant the testing of recombinant MSP1 and the other recombinant malaria vaccines under development in CpG ODN plus Montanide adjuvant formulations in human trials.

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